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In accordance with Task 1 of the Statement of Work, the HB4a cell line was characterized with respect to its requirements for normal cellular growth, and the conditions needed to support growth in soft agar. After determining the optimal conditions for supporting growth in soft agar, further effort was placed on optimizing the use of the human erbB2 expression vector. Because a large part of the experimental design included the use of single cell microinjection assays, there was a need to tag the erbB2 construct for ease of identification. Since erbB2 is a membrane protein, I was unwilling to attach a tag directly to the protein. Instead, an Ires-GFP element was placed downstream of erbB2 in the pcDNA background. Transient transfection and microinjection studies confirmed the expression from the Ires-GFP element in HB4a cells. Microinjection experiments were performed using both MnSOD and erbB2-Ires-GFP in HB4a cells to identify the role of MnSOD in mediating the proliferative response to erbB2. The results initially showed a MnSOD mediated inhibition of erbB2 induced cell cycle progression. However, a recent analysis of the dual expression of MnSOD and erbB2 indicated a significant level of promoter competition between the 2 constructs. Current efforts are focused on subcloning the MnSOD cDNA into an expression vector that has been shown to work well with the pcDNA promoter. Once this issue is resolved, I will repeat the analysis of cell cycle progression.and continue with Tasks 1 and 2 as planned.

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Introduction

An investigation into the role of Manganese Superoxide Dismutase (MnSOD) in EGFR positive breast cancer development began within the past year, with the help of funding provided by this grant. Results generated prior to the receipt of funding indicated that while low expression of MnSOD appeared to inhibit NIH3T3 cellular transformation in response to constitutive activation of the Ras pathway, higher levels of MnSOD expression appeared to potentiate Ras induced cellular transformation. The initial goal of this grant was to demonstrate that such a dual role for MnSOD in mediating cellular transformation via the Ras signaling pathway may be relevant to the development of EGFR overexpressing breast cancer. In pursuit of that goal, we have begun to establish the tools needed for a thorough analysis of this issue. As discussed below, progress has been made in establishing the proper system for analyzing MnSOD's effect on EGFR positive breast cancer, and preliminary results have pointed to a possible role for MnSOD in EGFR mediated cell cycle progression.

Body

Initial work focused on Task 1 of the approved Statement of Work, specifically, establishing the proper conditions for working with the breast epithelial cell line, HB4a. The cells were initially received from Dr. Michael J O'Hare (Ludwig Institute for Cancer Research), and immediately tested for their ability to grow in soft agar. Problems were initially encountered in establishing the growth in soft agar assay. Early attempts at characterizing the growth in soft agar properties of the HB4a cell line repeatedly resulted in a dissolution of the soft agar support. Consultation with Dr. Gregory Hannon's lab (Cold Spring Harbor Laboratory) yielded technical improvements, and the protocol was optimized to include a 0.5% Noble Agar bottom layer, a 0.3% Noble Agar top layer, and weekly replenishment of EGF ligand in a 0.3% Noble Agar matrix. This assay system was then used to analyze the growth properties of HB4a cells in response to the ectopic expression of the EGFR family member, erbB2. Preliminary growth in soft agar experiments have demonstrated the ability of erbB2 to transform the HB4a cell line by transient transfection at levels as low as 2 μg per 60mm confluent dish. Work is currently in progress to quantitate the transformation induced by increasing amounts of erbB2 in the HB4a cells, such that the optimal condition may be used in MnSOD co-expression assays.

Concurrent with efforts to characterize the growth in soft agar assay, an effort was undertaken to optimize the erbB2 gene product we will be using throughout the course of this experimental design. Dr. Yosef Yarden (The Weizmann Institute of Science) supplied us with a human erbB2 cDNA in the pcDNA 3.0 expression vector. Because of the use of single cell, microinjection assays in Task 1 of the approved Statement of Work, we decided that it was necessary to add a tag onto the erbB2 cDNA in order to allow for ease of visualization. However, since erbB2 contains a transmembrane domain, we were reluctant to attach a tag directly to the receptor itself. Instead, the alternative approach of placing an Ires-GFP element downstream of the erbB2 cDNA (but upstream of any

polyA signals), was undertaken. An Ires-GFP element was obtained from Dr. Scott Lowe (Cold Spring Harbor Laboratory), and was successfully placed within the pcDNA expression vector, downstream of erbB2. The creation of this erbB2-Ires-GFP construct results in the transcription of a bicistronic mRNA, from which erbB2 and GFP can be independently translated into protein. The advantage of this construct is that while the GFP will indicate in which cells erbB2 is expressed, it is a distinct protein that will not interfere with the folding, localization, or function of erbB2. Transient expression of the erbB2-Ires-GFP construct in the HB4a cell line demonstrated successful transcription and translation of the GFP reporter (Figure 1).

After establishing the functionality of the erbB2-Ires-GFP construct, we attempted to perform the microinjection studies outlined in Task 1. In order to assess the role of MnSOD in erbB2 mediated cell cycle progression, we made use of the SRE-CAT reporter assay described in Specific Aim 1. This assay reflects the activation of the MAP Kinase pathway by directly reporting Erk mediated transcription at the serum response element (SRE). HB4a cells were microinjected with high levels of erbB2 (75ng/μL) in the presence or absence of MnSOD. The cells were kept in a serum starved state, and incubated at 37°C for 36 hours. The cells were then fixed and stained for chloramphenicol acetyl-transferase (CAT) expression. This preliminary experiment demonstrated an induction of CAT expression by erbB2 overexpression alone, with no induction of CAT expression when MnSOD was coexpressed (data not shown). Similar results were obtained when a BrdU incorporation assay was performed to assess the effect of MnSOD on erbB2 mediated cell cycle progression (data not shown). These preliminary results appear to be in agreement with earlier work from our lab demonstrating an ability of MnSOD to suppress H-ras V12 mediated transformation. We will repeat these microinjection experiments with increasing levels of MnSOD to identify whether or not higher levels of MnSOD may synergize with the erbB2 pathway to induce cell cycle progression. However, these results are preliminary, and since they were obtained, we have observed a problem with the co-expression of erbB2-Ires-GFP and MnSOD. We have recognized a significant level of promoter competition between erbB2-Ires-GFP and MnSOD, as well as between erbB2-Ires-GFP and the empty vector, pCGN, into which MnSOD was cloned. Introduction of either the MnSOD or pCGN vectors causes a qualitatively significant decrease in erbB2-Ires-GFP expression, as judged by GFP immunofluorescence. We are currently designing a strategy to subclone MnSOD out of the pCGN vector and into a vector known to cooperate better with the pcDNA vector. We anticipate a quick resolution of this promoter competition problem, following which we will repeat the microinjection studies, begin soft agar assays with varying concentrations of MnSOD, and move onto the studies outlined in Task 2 of the Statement of Work.

In addition to the work performed in accordance with Task 1 of the Statement of Work, we have begun work to create a small interfering RNA (siRNA) vector to suppress the expression of endogenous MnSOD. Because of the inducible nature of MnSOD in response to cellular stresses, it was evident that such endogenous signaling might interfere with efforts to ectopically control cellular MnSOD levels. The use of siRNA directed specifically against endogenous MnSOD will yield a cellular background devoid of MnSOD, from which we will be able to draw more confident conclusions about the different levels at which we will ectopically express MnSOD. This work has just

recently begun, and has resulted in the creation of several clones containing the siRNA insert, all of which need to be tested for their ability to suppress endogenous MnSOD expression.

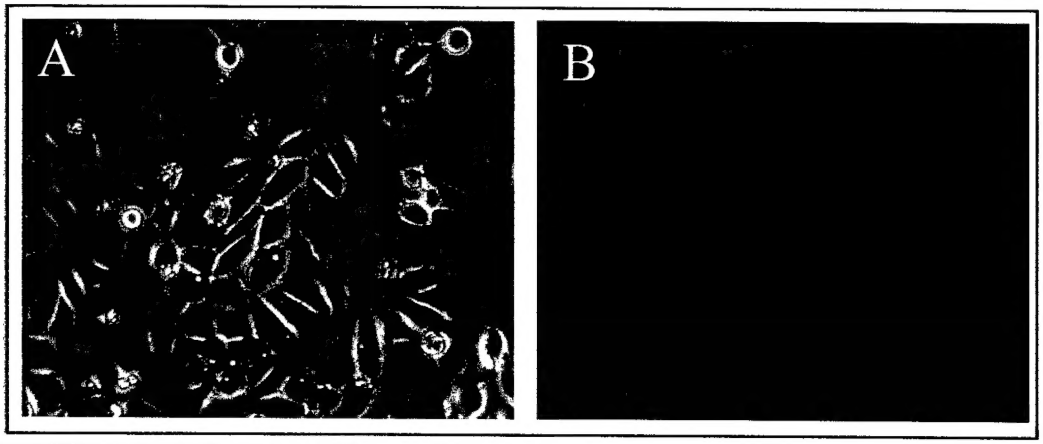

Figure 1: HB4a cells were transfected with erbB2-Ires-GFP DNA using a lipid based transfection method. Cells were visualized after 48 hours of expression under fluorescence microscopy. Panel (A) shows a field of HB4a cells under phase microscopy, and panel (B) shows that same field through a GFP fluorescence filter.

Key Research Accomplishments

1. Obtained and established conditions for HB4a cell line maintenance.
2. Optimized conditions for growth in soft agar assays using the HB4a cell line.
3. Generated the erbB2-Ires-GFP construct to allow for visualization of erbB2 expressing cells.
4. Established conditions for microinjection of HB4a cell line, and in preliminary experiments, demonstrated a suppression of erbB2 induced cell cycle progression by MnSOD.
5. Identified a problem of promoter competition between MnSOD and erbB2-Ires-GFP expression vectors, to be remedied by subcloning MnSOD into a new expression vector.

Reportable Outcomes

To date, this grant has not generated any reportable outcomes.

Conclusions

Work performed over the past year in accordance with the approved Statement of Work, has clearly demonstrated the feasibility of using the HB4a cell line for a study of the role of MnSOD in modulating erbB2 mediated breast cancer development. We have preliminary data suggesting that ectopically expressed erbB2 is capable of inducing cellular transformation (as judged by growth in soft agar) in the HB4a cell line. In

addition to the establishment of the growth in soft agar assay system to be used throughout the remainder of the experimental design, initial attempts at analyzing the relationship between MnSOD and erbB2 in the induction of cell cycle progression led us to discover a technical problem with our experiments. The identification of significant promoter competition between our MnSOD and erbB2-Ires-GFP constructs, has prevented us from making premature conclusions based on our microinjection studies, and has led us to redesign the MnSOD expression vector in order to avoid this problem throughout the rest of the study. The training value of this grant has been particularly high, due in large part to the problems that have been encountered. Encountering problems and devising strategies to solve them has been the notable utility of this grant as a method of pre-doctoral training.